Effect of mineral trioxide aggregate on cytokine production by peritoneal macrophages

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Abstract

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Aim To test the effect of two commercial brands of grey mineral trioxide aggregate (ProRoot® and MTA-Ângelus®) on cytokine production by M1 and M2 inflammatory macrophages.

Methodology M1 (from C57BL/6 mice) and M2 peritoneal inflammatory macrophages (from C57BL/6 IL12p40$^{-/-}$ mice) were obtained and cultured *in vitro* in the presence of MTA. The cellular viability and the production of tumour necrosis factor-α, interleukin (IL)-12 and IL-10 in response to stimulation with interferon-γ and *Fusobacterium nucleatum* or *Peptostreptococcus anaerobius* were evaluated. Data were analysed by Mann–Whitney, Kruskal–Wallis and ANOVA tests.

Results The cements did not interfere with cellular viability or with cytokine production by either type of macrophage. However, M2 macrophages produced higher levels of IL-10 when stimulated with *F. nucleatum* than M1 macrophages ($P < 0.05$).

Conclusions The brands of MTA evaluated did not interfere in the cytokine response by M1 or M2 macrophages to the two bacteria tested. However, a difference in cytokine production between the two types of macrophages was found.

Keywords: biocompatibility, cytokines, innate immunity, macrophages, mineral trioxide aggregate.

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Introduction

Since its first description (Lee *et al.* 1993), mineral trioxide aggregate (MTA) has been used in a variety of surgical and nonsurgical endodontic applications. Research has demonstrated that MTA has better properties in terms of root repair and bone formation, when compared with other commonly used materials such as intermediate restorative material (IRM®; Dentsply, Milford, DE, USA), glass ionomer and reinforced zinc oxide–eugenol cement (Super-EBA®; Harry J. Bosworth Company, Skokie, IL, USA) (Torabinejad *et al.* 1995a, 1998). As MTA is usually applied on infected or sterile surgical wounds, it is important that it is biocompatible and does not interfere with the immune response of the patient.

Inflamed periradicular and pulp tissues, upon which MTA is generally applied, present a variety of immunocompetent cells. Macrophages are the predominant cells (Stern *et al.* 1981, Kawashima *et al.* 1996) at the site. These cells play a central role in the pathogenesis of inflammation (Van Furth *et al.* 1972, Unanue 1978) and cytokine production is one among their several actions.

Recent studies have revealed that there may be three types of macrophages, based on their ability to produce different responses (Mosser 2003). As defined by Mills *et al.* (2000), M1 macrophages (obtained from C57BL/6 mice) will utilize arginine to produce nitric oxide (NO), while M2 macrophages (obtained from BALB/c mice) will produce low amounts of NO and high amounts of TGF-β. In addition, M1 macrophages are

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high producers of oxygen reactive species (Mills *et al.* 2000, Mosser 2003), while there is evidence that M2 macrophages do not produce interleukin (IL)-12 but release high amounts of IL-10 (Gerber & Mosser 2001). A third type, defined as the alternatively activated macrophage, converts arginine into ornithine by action of arginase. Ornithine is the precursor of polyamines that will mediate collagen synthesis and cellular proliferation. Hence, M1 macrophages (obtained from C57BL/6 mice) would be high producers of IL-12 and low producers of IL-10, in accordance with their pro-inflammatory role, while M2 macrophages (obtained from BALB/c mice) would produce lower levels of IL-12 and higher levels of IL-10 and TGF-β, which would render them an anti-inflammatory role (Mills *et al.* 2000, Gerber & Mosser 2001, Mosser 2003). Recently, Bastos *et al.* (2002) showed that mice deficient in the p40 chain for IL-12/IL-23 by targeted mutation have predominantly macrophages of the M2 type, like the BALB/c mice. This model is elegant, as it provides macrophages of the same genetic background (in this case the C57BL/6) but with different phenotypes (M1 or M2).

As MTA has only recently been used as an endodontic material, there are few studies evaluating its effects on the host defense mechanisms (Koh *et al.* 1997, 1998, Mitchell *et al.* 1999). This study aimed to analyze the action of two commercial brands of MTA (MTA-Ângelus®, Odonto-lógika, Londrina, PN, Brazil; ProRoot®, Dentsply Maillefer, Ballaigues, Switzerland) on the activity of M1 and M2 murine macrophages, assessing cellular viability and cytokine production.

Materials and methods

Preparation of MTA

Two different commercial brands of grey MTA were used: ProRoot® (Dentsply Maillefer, Ballaigues, Switzerland) and MTA-Ângelus® (Odonto-lógika, Londrina, PR, Brazil). The cements were prepared according to the manufacturer's specifications, and their manipulation was carried out in a laminar flow hood under aseptic conditions. Cements were inserted into the ends of capillary tubes (Mícron, Trianon Ind. e Com. Ltda, São Paulo, SP, Brazil), previously cut and sterilized (capillary diameter: 3.52 mm^2), according to the technique described previously (Oliveira Mendes *et al.* 2003). Capillaries containing cements were stored in six well culture plates (Nunclon; Nalge Nunc International, Naperville, IL, USA) for final setting. Subsequently, the plate was sterilized using 25 kGray of gamma ray irradiation (Centro de Desenvolvimento Tecnológico/CDTN, Belo Horizonte, MG, Brazil).

Mice

Males and females of 4–8 weeks of two mouse strains were used: C57BL/6 (CEBIO – UFMG, Belo Horizonte, Brazil) and C57BL/6 deficient in the p40 chain for IL-12/IL-23 by targeted mutation (Magram *et al.* 1996). Matrices for IL-12p40$^{-/-}$ were obtained from Dr Luiz Vicente Rizzo (University of São Paulo, SP, Brazil), mice were bred at the Gnotobiology Laboratory, UFMG, Belo Horizonte, Brazil. Experimental animals were kept in a conventional animal house with barriers, temperature and light control. Food and water were offered *ad libitum*. The research protocol was authorized by the committee of ethics and research on animals (CETEA – UFMG, protocol number 35/2002).

Macrophages

Inflammatory macrophages were obtained from the peritoneal cavity, 5 days after injection of 2 mL of 3% thioglicolate medium, containing 1% sterile agar (Biobrás S.A., Montes Claros, MG, Brazil). The animals were then sacrificed and 10 mL of sterile RPMI 1640 medium, without phenol red (Sigma Chemical Co., St Louis, MO, USA), was injected into the peritoneal cavity. The largest possible content was aspirated, and the cells were centrifuged at 350 *g* for 10 min, at 4 °C. Supernatant medium was discarded and cells were resuspended in RPMI 1640 medium without phenol red, supplemented with 10% fetal bovine serum (Nutricell; Campinas, SP, Brazil), 0.05 mmol L^{-1} β-mercaptoethanol (Sigma Chemical Co.), 0.2% gentamicin and 200 mmol L^{-1} L-glutamine. The cells were counted in a Newbauer chamber, and the final concentration was adjusted for each experiment.

Viability

To evaluate the cellular viability in the presence of MTA, the capillaries containing MTA and empty controls were added onto the cellular suspension (1×10^5 cells mL^{-1}) present in 24-well plates (Nunclon). Cells were incubated for 24, 48 or 72 h. The viability analysis was accomplished with an inverted microscope, following the addition of 100 μL of 0.5% tryptan blue solution (Sigma Chemical Co.) in PBS (0.15 M NaCl and 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4).

Live and dead cells were counted, amounting to at least 300 cells (Oliveira Mendes *et al.* 2003).

Bacterial preparations

To induce the production of tumour necrosis factor (TNF), IL-12 and IL-10, *Fusobacterium nucleatum* (ATCC 10953) and *Peptostreptococcus anaerobius* (ATCC 27337) were prepared, and used in a proportion of 10 UFC for each macrophage in culture (Ribeiro Sobrinho *et al.* 2002). These preparations were used to stimulate cell cultures.

Cell cultures and cytokine assays

Fusobacterium nucleatum and *P. anaerobius* (10^7 UFC mL^{-1}) preparations were added to the 24-well plates (Nunclon), containing 1 mL of peritoneal cell suspension (2×10^6 cells mL^{-1}), in the presence or absence of 10 U mL^{-1} of recombinant murine interferon (IFN)-γ (Pharmingem, San Diego, CA, USA), in addition to the MTA-containing or empty capillaries. Plates were incubated for 24 h for TNF and IL-12 assays, and for 72 h for IL-10. Supernatants were harvested, and cytokine readings were carried out (Duo Set Elisa kits for TNF, IL-12p70 and IL-10; Development System, R & D Systems, Mineapolis, MN, USA). Sensitivity for the assays were as follows: TNF: 31.2 pg mL^{-1}; IL12p70: 23.4 pg mL^{-1}; IL-10: 15.6 pg mL^{-1}.

Statistical analysis

Results were compared by ANOVA, Mann–Whitney and Kruskal–Wallis tests as specified for each experiment. The analyses were carried out with SPSS 8.0 Inc. software (Statistical Package for Social Sciences, Chicago, Ill, USA).

Results

Cell viability in the presence of MTA

The results of cellular viability are shown in Fig. 1. The mean cellular viability was >97% in all the M1 and M2 macrophage cultures, regardless of exposure to MTA. There was no difference in the percentages of live cells between the two cements. However, the proportion of M2 macrophage live cells exposed for 24 h to MTA-Ângelus was statistically higher than the M1 macrophages ($P < 0.05$). The incubation time did not influence the percentage of live M1 macrophages. M2 macrophages were less viable after 72 h of culture ($P < 0.05$). However, as viability was >97% in all cultures, cytokine production in these cultures was investigated.

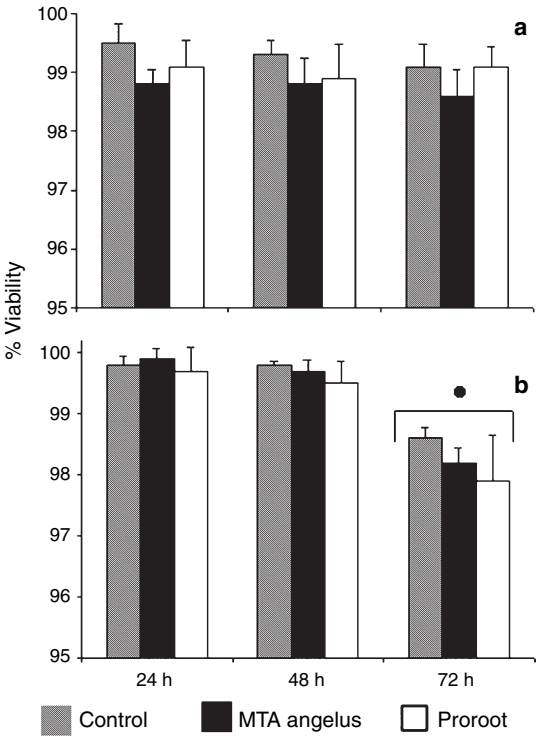

Figure 1 Percentage of live M1 (a) and M2 (b) macrophages 12, 24 and 48 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the means. • indicates $P < 0.05$ when compared with 24 and 48 h by Kruskal–Wallis tests.

TNF-α

Inflammatory M1 and M2 macrophages produced TNF-α in culture in the absence of bacterial stimuli (Fig. 2). TNF-α production by M1 or M2 was not influenced by the presence of either brand of MTA. M1 macrophages produced TNF-α in response to all stimuli. However, because of variability among experiments and the consequent high standard deviations, statistical differences were found only among the control cultures and the cultures stimulated with *P. anaerobius* and IFN-γ. M2 macrophages stimulated with IFN-γ and either *F. nucleatum* or *P. anaerobius* produced higher levels of TNF-α than nonstimulated cultures.

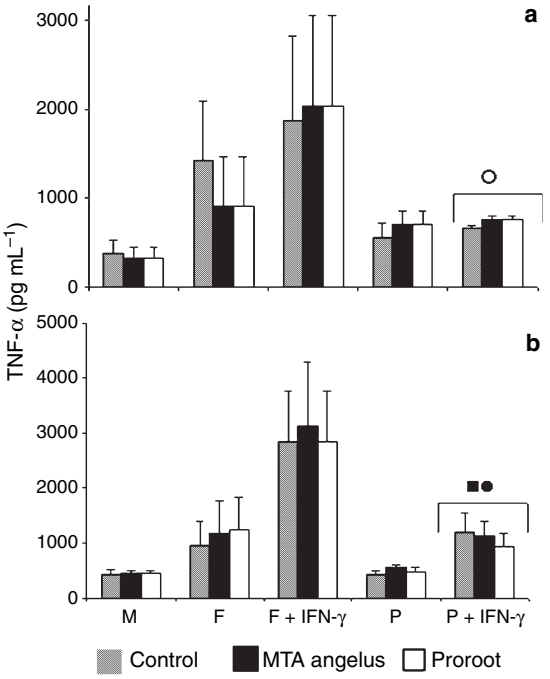

Figure 2 Production of tumour necrosis factor-α by M1 (a) and M2 (b) macrophage cultures 24 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Cells were cultured in the medium alone (M) and *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon (IFN)-γ (I) was added where indicated at 10 units mL^{-1} (1 μg = 8430 units). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the means. ○ indicates $P < 0.05$ by Kruskal–Wallis test when compared with macrophages in medium alone. ● and ■ indicates $P < 0.05$ when compared with bacterial preparations without IFN-γ.

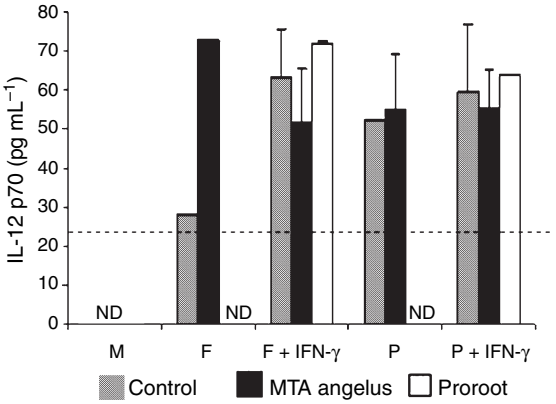

Figure 3 Production of interleukin (IL)-12p70 by M1 macrophage cultures 24 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Cells were cultured in the medium alone (M) and *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon-γ (I) was added where indicated at 10 units mL^{-1} (1 μg = 8430 units). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the mean values; - - - indicates sensitivity (23.4 pg mL^{-1}) of the assay; ND, not detected.

IL-12p70

Production of IL-12p70 was undetected in several conditions, and, when detected, was just above the detection limit of the assay. Neither brand of MTA influenced the production of this cytokine by M1 macrophages. Consistent IL-12 production was found when cells were stimulated with both bacterial preparations and IFN-γ (Fig. 3).

IL-10

Once again, neither brand of MTA influenced the production of cytokine by M1 or M2 macrophages (Fig. 4). Neither type of macrophage produced IL-10 in the absence of bacterial stimuli. M1 macrophages produced IL-10 in response to all stimuli, and addition of IFN-γ did not influence the levels of IL-10 produced. *Fusobacterium nucleatum* induced significantly higher levels of IL-10 production by M2 macrophages than *P. anaerobius.* IFN-γ inhibited the production of IL-10 stimulated by *F. nucleatum.* Moreover, *F. nucleatum*-stimulated M2 macrophages produced higher levels of IL-10 than *F. nucleatum*-stimulated M1 macrophages.

Discussion

Since its introduction as an endodontic cement (Lee *et al.* 1993), MTA has been studied both *in vivo* (Holland *et al.* 2002, Saidon *et al.* 2003) and *in vitro* (Koh *et al.* 1998, Osorio *et al.* 1998, Mitchell *et al.* 1999, Zhu *et al.* 2000, Abdullah *et al.* 2002, Haglund *et al.* 2003). These studies have demonstrated that MTA has excellent biocompatibility, causing no significant tissue inflammation at the site of its application. It allows repair, inducing the deposition of dentinal (Tziafas *et al.* 2002), cemental (Torabinejad *et al.* 1995b, Holland *et al.* 2001) and bony tissues (Torabinejad *et al.* 1995b, 1998).

It is known that during the pulp inflammatory process, an array of immunocompetent cells is attracted to the site in an attempt to eliminate the aggressor

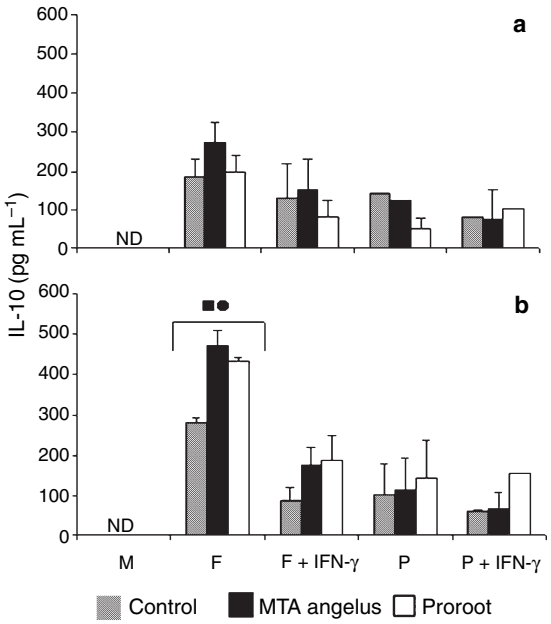

Figure 4 Production of interleukin-10 by M1 (a) and M2 (b) macrophage cultures 72 h after incubation in the presence of mineral trioxide aggregate (MTA)-Ângelus, MTA ProRoot or empty capillaries (control). Cells were cultured in the medium alone (M) and *Fusobacterium nucleatum* (F) or *Peptostreptococcus anaerobius* (P) preparations. Interferon-γ (I) was added where indicated at 10 units mL^{-1} (1 μg = 8430 units). Bars represent the mean of three experiments carried out independently in duplicates and lines represent the standard deviations of the mean value. ■ indicates $P < 0.05$ for M2 macrophages stimulated with *F. nucleatum* compared with all other conditions by ANOVA followed by Tukey's multiple comparisons. ● indicates $P < 0.05$ when M1 and M2 macrophages stimulated with *F. nucleatum* were compared by ANOVA test followed by Tukey's multiple comparisons.

stimulus. Macrophages are among the first cells to come in contact with foreign bodies and play the main role in the pathogenesis of the inflammatory process (Van Furth *et al.* 1972, Unanue 1978). Some authors (Yu & Stashenko 1987) believe that lymphocytes are the most prevalent cells in the periapical inflammatory infiltrate whereas others believe that macrophages are most prevalent (Stern *et al.* 1981, Kawashima *et al.* 1996). Macrophages produce several cytokines that promote the initiation, perpetuation, directing and the inhibition of the immunological response process (Stashenko *et al.* 1998, Metzger 2000).

In this study, macrophages were exposed to manipulated MTA *in vitro*, using a previously described system (Oliveira Mendes *et al.* 2003) that allows a controlled exposure of cell cultures to the cement. In these conditions, viability was >97% at all periods examined, and was similar in the presence of the two commercial brands; however, it was lower for the M1 macrophages in contact with MTA-Ângelus, for 24 h. This difference was not observed in the subsequent periods. The high cellular viability in the presence of MTA coincide with that for fibroblasts (Mitchell *et al.* 1999, Keiser *et al.* 2000, Saidon *et al.* 2003) and osteoblasts (Koh *et al.* 1997, Mitchell *et al.* 1999). Only one study was found in the literature on the viability of macrophages exposed to MTA (Haglund *et al.* 2003). In that paper, the authors observed a normal morphology of macrophages in contact with dry MTA. MTA also allowed increased viability when compared with the other materials evaluated. However, lower cell numbers were found in cultures exposed to MTA when compared with nonexposed controls. A very large area of each material was exposed to cells in culture, and, perhaps, this was the reason for the higher mortality in MTA-exposed cultures when compared with controls, in that study.

The decrease in viability of the M2 macrophages in culture is intriguing. No data are available in the literature as to differences in viability of M1 and M2 macrophages in culture. It is interesting that a cell type that is supposedly not producing IFN-γ (Mosser 2003) and lower levels of NO (Mills *et al.* 2000, Bastos *et al.* 2002) than M1 dies more easily. However, the decrease in viability was small. In the present study, the 72-h cultures were used to detect IL-10. Thus, it is possible that slightly higher values of this cytokine could be detected in M2 cultures, if the cells were completely viable.

Periapical inflammation is not only the result of the direct action of microorganisms, but also of pro-inflammatory mediators, like the cytokines (Stashenko *et al.* 1998). These mediators act on the innate immunity, antigen presentation, differentiation within the bone marrow, cell recruiting and activation, and adhesion molecule expression (Borish & Steinke 2003). The present study has investigated the production of TNF-α, IL-12 and IL-10 by macrophages exposed *in vitro* to MTA, as these cytokines are involved in the onset of the inflammatory process (TNF-α), the interconnection of the innate and adaptive immune responses (IL-12), and the regulation or the inflammatory process (IL-10). In addition, TNF-α is an important mediator in the activation of macrophages is osteoclastic activation factor. IL-12 and IL-10 are macrophage polarization markers. Addition of bacterial

antigens and IFN-γ to the cell cultures was an attempt to reproduce the clinical conditions, where the presence of anaerobe bacteria (Sundqvist 1992), as well as the presence of other cytokines (Stashenko *et al.* 1998) is a frequent finding. IFN-γ acts by stimulating antigen presentation and cytokine production by macrophages and dendritic cells, in addition to activating the effector functions of the macrophages. These functions include adherence, phagocytosis, secretion, respiratory explosion and the production of nitric oxide (Borish & Steinke 2003).

Tumour necrosis factor-α is mainly produced by mononuclear phagocytes with the purpose of stimulating neutrophil and monocyte recruitment to the infection site, and activating these cells to eradicate microorganisms. In apical lesions, TNF-α, IL-1-α, IL-1-β, TNF-β, IL-6 and IL-11 act on bone resorption, and they collectively constitute the osteoclastic activation factor (Stashenko 1990). In the present study, large amounts of TNF-α were observed following incubation for 24 h with bacterial preparations and with these preparations and IFN-γ. The production of TNF-α by macrophages was also observed when in contact with the capillaries with and without the MTA, which is not surprising given that macrophages were harvested from the peritoneal cavity of mice after an inflammatory stimulus. Hence, MTA did not affect the capacity of macrophages to produce TNF-α on response to triggering stimuli.

Interleukin-12 induces the production of IFN-γ by natural killer cells and T cells, a fact that adds to the activation of phagocytic cells, in addition to favouring the differentiation of T-helper cells into the Th1 subset. In addition to these actions, it works as a link between the innate and the adaptive antigen-specific responses (Trinchieri & Scott 1995). We observed that the production of IL-12 increased both in presence of gram-positive and gram-negative bacteria, and that the addition of IFN-γ seemed to lead to an even greater IL-12 production (however, no statistical significance was found when cultures with and without addition of IFN-γ were compared). These data are in accordance with those reported in the literature (Ma *et al.* 1996, Hayes *et al.* 1998). Again, MTA did not affect the capacity of macrophages to produce IL-12.

Interleukin-10 induces tissue homeostasis, leading to the inhibition of pro-inflammatory cytokine production by activated T cells and macrophages (Kawashima *et al.* 1996, Stashenko *et al.* 1998, Gerber & Mosser 2001, Borish & Steinke 2003). Both *F. nucleatum* and *P. anaerobius* triggered IL-10 production. *Fusobacterium nucleatum* stimulated M2 macrophages to produce higher levels of IL-10 than M1 macrophages. This is in accordance with the literature, which shows that M2 macrophages are high producers of IL-10 (Mosser 2003). However, *P. anaerobius* did not stimulate M1 and M2 differently. For some time it had been believed that gram negative bacteria would be better inducers of IL-10 than gram positive bacteria (Hessle *et al.* 2000, Cross *et al.* 2004). Here, this observation was confirmed: the gram negative bacterium (*F. nucleatum*) stimulated more IL-10 than the gram positive (*P. anaerobius*). None of the studies in which the gram positive/gram negative, IL-12/IL-10 dichotomies were addressed used mouse-derived M1 or M2 macrophages. In one study human monocytes were used (Hessle *et al.* 2000), in the other, a macrophage cell line (Cross *et al.* 2004). Hence, our results show that for M2 macrophages a gram negative bacterium induces more IL-10 than a gram positive bacterium. The same was not found for the low IL-10 producing M1 macrophages. Also of note is the fact that IFN-γ down-modulated production of IL-10 only by *F. nucleatum*-stimulated M2 macrophages. In short, it was found that the two different types of macrophages have only differed as to the production of IL-10 (besides the obvious lack of IL-12 production by the population of M2 used here), which is in agreement with previous reports (Mosser 2003).

It has been shown that MTA did not affect the production of the cytokines, either alone, or in conjunction with gram positive or gram negative bacteria and IFN-γ. This appears to be the first report in which the effect of MTA on the production of polarizing cytokines by M1 and M2 macrophages has been determined. One other report on the effect of MTA on cytokines that mediate bone resorption has shown that this cement does not, by itself, induce the production of these cytokines (Haglund *et al.* 2003). However, it should be borne in mind that the compatibility of MTA demonstrated here and elsewhere (Koh *et al.* 1997, Haglund *et al.* 2003, Saidon *et al.* 2003) has all the limitations of *in vitro* work. Additional work should be carried out to assess the effects of MTA *in vivo*, as cytokine synthesis *in vivo* is a very complex event, and its expression and effects are ruled by many factors, including other cells and mediators (Mitchell *et al.* 1999).

Conclusion

Mineral trioxide aggregate had excellent results concerning macrophage viability, and noninterference

with the production and secretion of the cytokines TNF, IL-12 and IL-10, by M1 and M2 macrophages.

Acknowledgements

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